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EXPERIMENTAL INFECTION WITH *STREPTOMYCES MADURAE* AS A FUNCTION OF COLLAGENASE*

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The enzyme collagenase occurs rarely among the fungi and bacteria. In reviewing the subject, Mandl (1) in 1961 concluded that only the clostridial (*Clostridium perfringens* and *C. histolyticum*) enzyme was an authentic collagenase. The criteria she proposed for distinguishing an enzyme as a specific collagenase were the reduction in viscosity of undenatured collagen and the concomitant release of dialyzable hydroxyproline-containing units at physiologic pH. Previous work by the senior author (2) has described enzymes elaborated by an actinomycete, (*Streptomyces madurae*) and a fungus, (*Trichophyton schoenleinii*) which fulfilled these criteria for collagenase. Schoellman (3) has recently described a collagenase from strains of *Pseudomonas aeruginosa.* This report is concerned with the possible significance of the enzyme to the pathogenic potential of the actinomycete *Streptomyces madurae,* an etiologic agent of human mycetoma or maduramycosis.

MATERIALS AND METHODS

Organism. S. madurae isolated from a case of mycetoma was maintained on trypticase soy agar (TSA) as stock culture. Organisms for animal inoculation and exposure to mutagen were grown in trypticase soy broth with 0.2% Tween 80 (TSBT) for two weeks at 25° C. The Tween was required to obtain single cells.

Collagenase assay. Preparation of substrate, and the assays for viscosity and hydroxyproline were previously described (2). A modification of the ninhydrin method of Mandl (1) was used for preliminary testing. To 10 mg collagen (Worthington) were added 5 ml 0.067 M phosphate buffer (pH 7.4) with 0.45% NaCl and 0.1 ml of enzyme solution (usually 0.1 mg/ml concentration). The mixture was incubated at 37° C overnight on a Dubnoff shaking water bath. After filtration, 1 ml of filtrate was added to 0.5 ml cyanide acetate buffer and 0.5 ml ninhydrin solution (4). This material was heated for fifteen minutes in a boiling water bath and then 5 ml of diluent (Isopropyl alcohol water 1:1) was immediately added. The solution was cooled and the developed color read at 570 mμ in a Beckman DU spectrophotometer. The results were expressed as liberated micromoles amino acid, equivalent to leucine.

Production of mutants. N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was used to induce mutants by a method modified from Clutterbuck and Sinha (5). The organisms as single cells were harvested from TSBT broth, washed three times in saline and resuspended in 2 ml Tris maleic (TM) buffer (M/20 adjusted to pH 6.0 with NaOH). The cell count was approximately 10^8 viable units per ml. Six mgm NTG were dissolved in 10 ml Tris maleic buffer. The "reaction" mixture consisted of: 2 ml cell suspension, 10 ml TM buffer containing the 6.0 mgm NTG (final concentration 0.5 mg/ml). The mixture was incubated at 37° C with slow agitation in a Dubnoff shaker. Aliquots (1 ml) were removed at various time intervals, washed three times with saline and placed on 140 mm petri dishes of trypticase soy agar. The percentage of survival as a function of exposure time was determined. Plates with 25% or less survivors were examined for mutants. At least thirty colonies were picked at random. Each clone was examined for all morphological and physiological characteristics as defined for the species *S. madurae* in Bergey's manual and each clone was examined for the production of collagenase. Mutants of *S. madurae* that elaborated no collagenase were significantly less pathogenic to mice.

Enzyme-deficient organisms that by all other criteria were identical to the wild type organism were tested for reversion to collagenase production and pathogenicity. The mutants were grown in TSBT broth harvested and treated with NTG as previously described. Colonies were randomly picked and assayed for collagenase. Clones that produced the enzyme in amounts equal to, less than, or greater than the wild type were tested in mice for pathogenicity.

The standard procedure for production of collagenase consisted of inoculation of 10^3 cells into 100 ml of TSB contained in 250 ml Erlenmeyer flasks. The flasks were incubated at 25° C in a shaking water bath for 14 days. The cells formed granules of similar morphology to that produced in tissue. The microcolonies were filtered off, washed

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FIG. 1. Subcutaneous lesion in mouse by day 12 following injection of 50 mg micro-colonies of *S. madurae* and 1 mg cortisone.

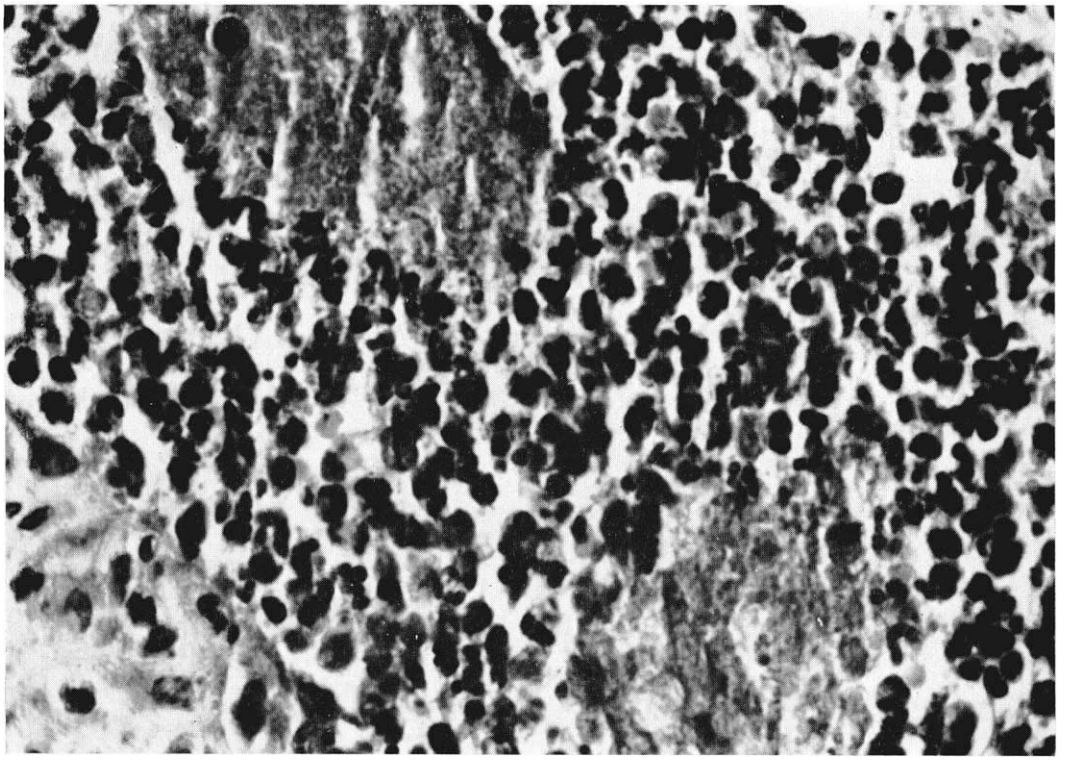

FIG. 2. Tissue response and granule formation in mouse on day 12. H & E stain

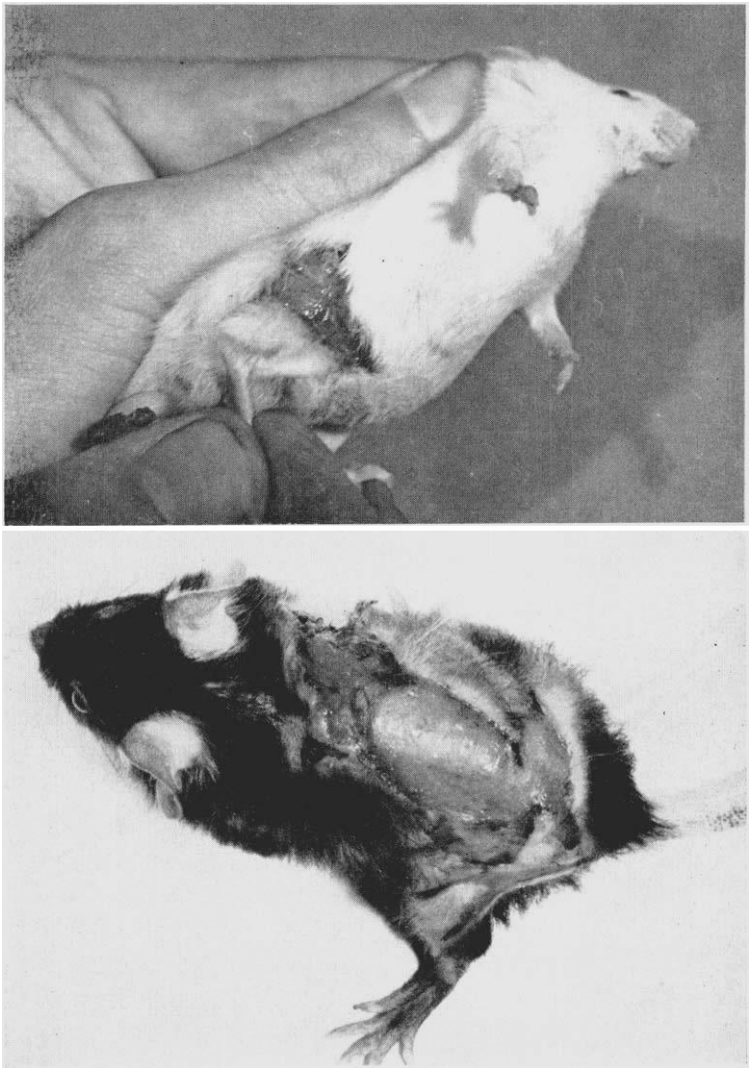

FIG. 3. Beginning of skin loss on day 16 in mouse injected with *S. madurae*
FIG. 4. Extensive skin loss in mouse on day 20 following injection of single cells of *S. madurae* (no cortisone).

in saline, centrifuged and weighed as wet buttons. The wild type *S. madurae* averaged 2.5 gms of cells in 100 ml. broth and all mutants were standardized to the same base. The culture filtrate was precipitated with ammonium sulfate added as a solid to a concentration of 40% and left over night in the cold. The precipitate was dissolved in distilled water, dialyzed against distilled water overnight and lyophilized. The enzyme powder was tested for collagenase activity by the ninhydrin method and production calculated on cell-weight basis. The enzyme produced by clones selected for further study was also tested for collagen viscosity reduction and hydroxyproline release.

Animal studies. Swiss albino and C-57 black, male and female mice were used for pathogenicity studies. No difference in the response of the strains or sexes was noted. All experiments employed sets of ten animals and each experiment was repeated at least three times.

Initially, microcolonies of *S. madurae* by weight (400, 200, 100, 50 and 25 mg) were injected into mice with and without concomitant cortisone (4, 3, 2, 1 and 0 mg/animal). The cortisone acetate (Upjohn) was given as a single injection i.p. and the organisms were injected into the right groin by the method of Macotela-Ruiz (6). It was later found that single cells grown in TSBT broth could be used with similar reproducible results and without the necessity of cortisone. Cells grown in TSBT

TABLE I

Summary of animal experiments with S. madurae *Disease production as a function of collagenase in* Streptomyces madurae

Criteria of Infection	Strains				
	Wild type* microcolonies	Wild type† 1×10^6	Enzyme-deficient†	Enzyme-excess†	Enzyme-revertant†
Day of onset of nodule	10	15	18‡	8-10	15-18
Day of skin loss	14-18	18-20	–	5	18-25
"Madura foot" formation	–	+	–	–	+
Tissue enzyme assay	+	+	–	+	+
Percentage survival to fourth month	40	10	95	0	10

* 50 mg microcolonies and 1 mg cortisone per mouse.

† Inoculum 1×10^6 single cells.

‡ Small nodules, all healed.

broth were washed three times in saline and resuspended to a concentration of 1×10^7 ml viable units or cells and 0.1 ml was injected into the right thigh or groin of each animal. The inoculated mice were examined at intervals for lesions and skin-loss.

Tissue assay for collagenase. Material from lesions was taken from infected mice at various times post inoculation. The material was weighed, minced, filtered, dialyzed and lyophilized. The powder was tested by the ninhydrin, viscosity and hydroxyproline assays for collagenase and activity per gram of tissue was calculated. Normal tissue from uninoculated mice and from mice inoculated with 10^6 dead organisms served as controls.

RESULTS

Cortisone treated mice. In mice given 1 mg of cortisone, 50 mg of *S. madurae* microcolonies would establish a consistently reproducible infection. Large subcutaneous swellings occurred at the injection sites by the tenth day. Histological examination showed a mixed pyogenic and granulomatous infection and the formation of 100–500 μ granules (Figs. 1, 2, 3). Between the 14th and 18th day, large areas of dermis separated from the underlying fascia and the mice recovered or died depending on the extent of denudement. Two hundred mgms. of organism as microcolonies were required to effect the same condition without cortisone (Table I).

Untreated mice. Growing the organism in TSBT broth produced single cells. Consistent infection was elicited when 1×10^6 viable units were injected. The course of the disease was similar to that for cortisone-treated animals; nodule formation was most pronounced at 15 days and skin-loss by 18–20 days (Figs. 4, 5). The histologic picture was also similar to the cortisone-treated animals (Figs. 6, 7). The granules appeared morphologically identical to those produced *in vitro* in trypticase soy broth (Figs. 8, 9). Skin loss occurred more slowly and eventually almost all mice died by the fourth month. Many animals developed a "madura foot"-like swelling in one or more feet (Fig. 10). Lesion material was found to contain enzyme activity (av. 285 μM/g). No activity was found in tissue from uninoculated mice, mice inoculated with dead organisms or mice inoculated with collagenase-deficient mutants (Table I).

Mutation for collagenase-deficiency. The effect of exposure of the organism to 0.5 mg/ml NTG is summarized in Table II.

Twenty randomly selected clones picked after the one hour exposure to NTG were tested for relative ability to elaborate collagenase (Table III).

Two clones that produced no collagenase were tested for relative disease producing ability. Small nodules were formed in most animals and granules were seen in biopsy. The lesions all healed by the twentieth day, no mice died and no mice suffered skin loss or "madura foot" formation (Table I). There was also no skin loss and little disease in animals injected with a low level (240 μM/mg) collagenase producing organism. Two animals out of ten died in this experiment.

One mutant that produced more collagenase than the wild type was also tested for its disease producing ability. The time of maximum nodule formation was shortened as was the survival time (Table I). Few of these animals lived to develop "madura foot".

One mutant clone deficient in collagenase

FIG. 5. Edge of denuded area on mouse with skin loss. Day 24. H & E stain

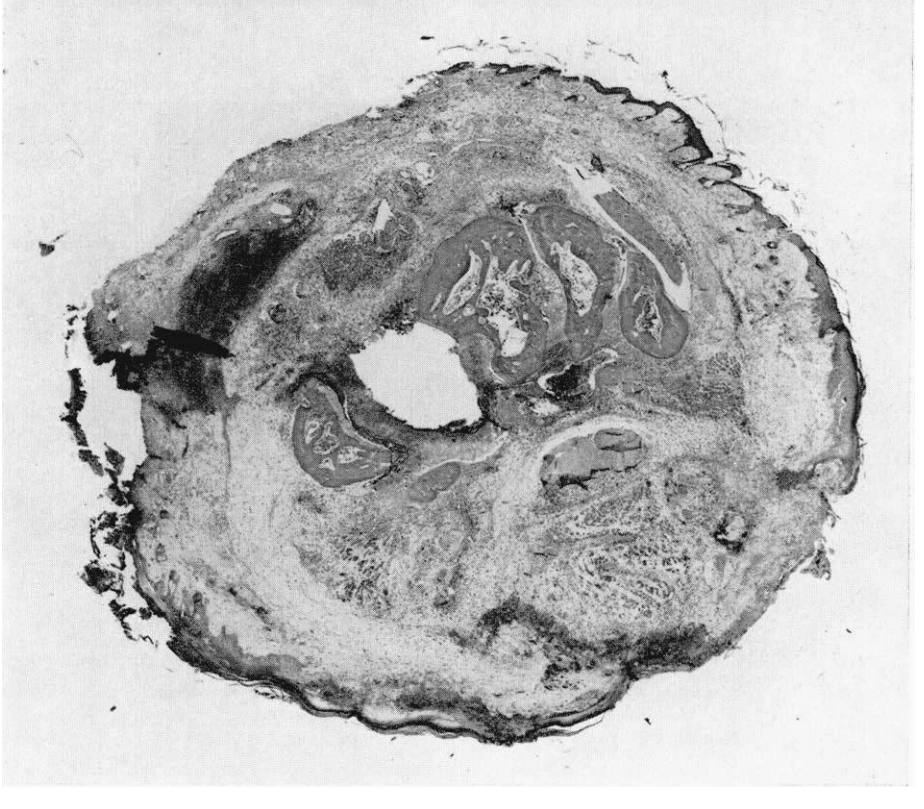

FIG. 6. Cross section of leg from "madura foot" of mouse showing invasion of deep tissue and sinus tract formation. Day 24. H & E stain.

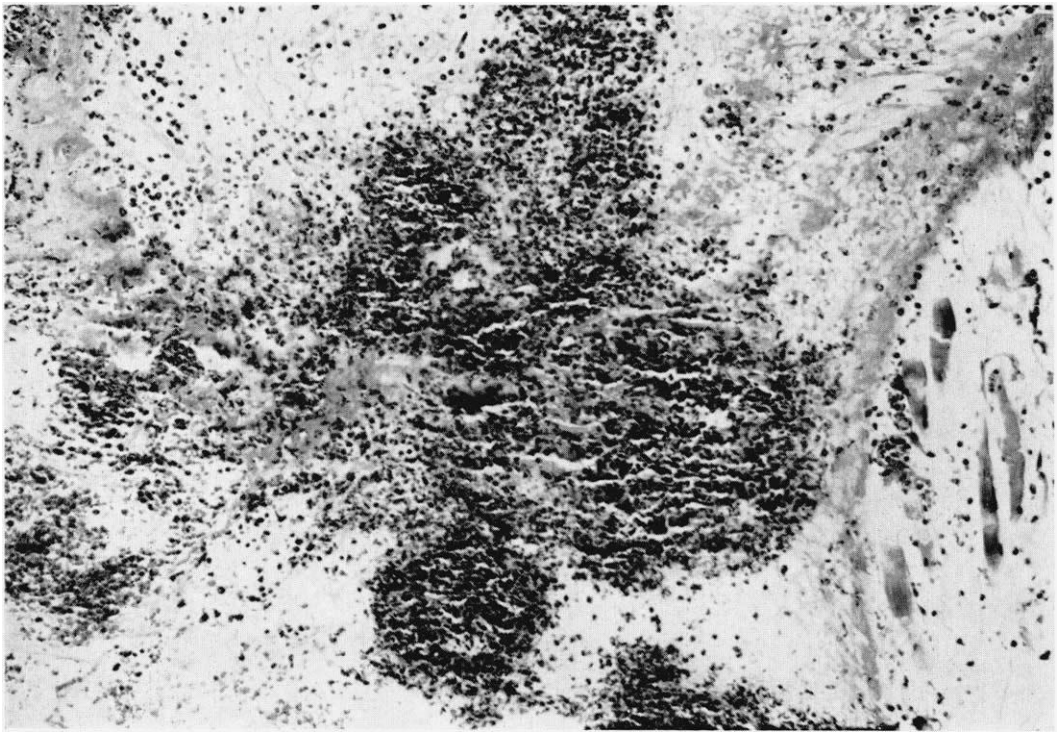

FIG. 7. Enlarged view of interior end of sinus tract with granules and pyogenic response. H & E stain.

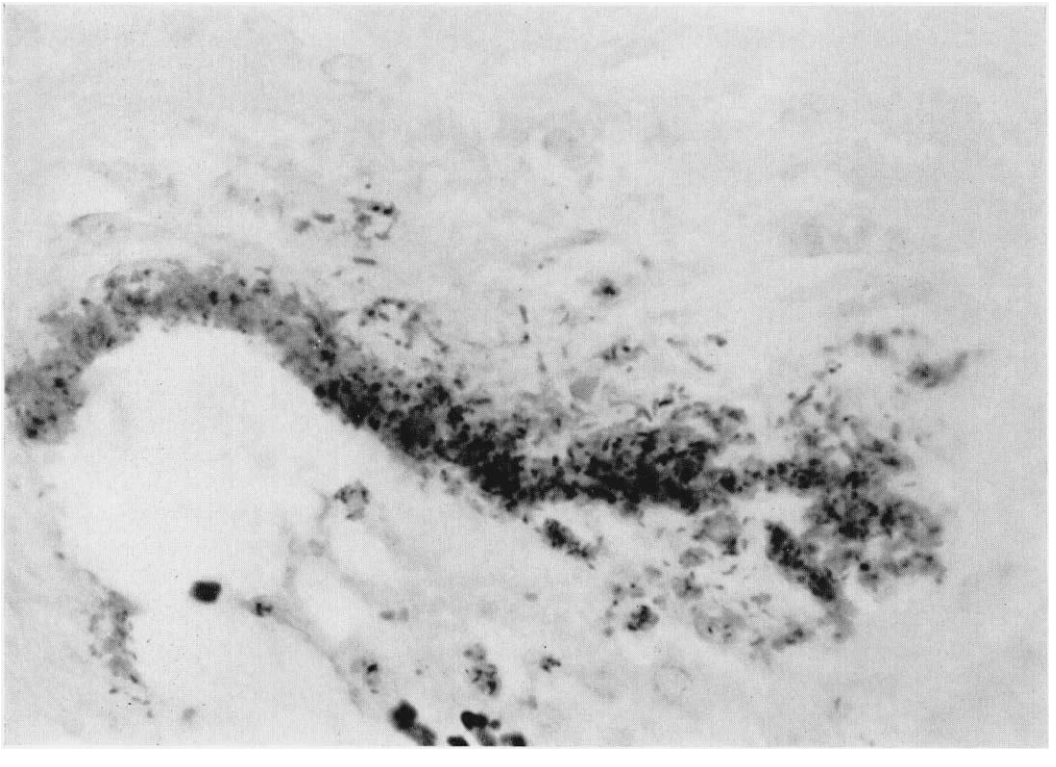

FIG. 8. Elongate granule running in sinus tract next to femur. Day 30. B. and B. Gram stain

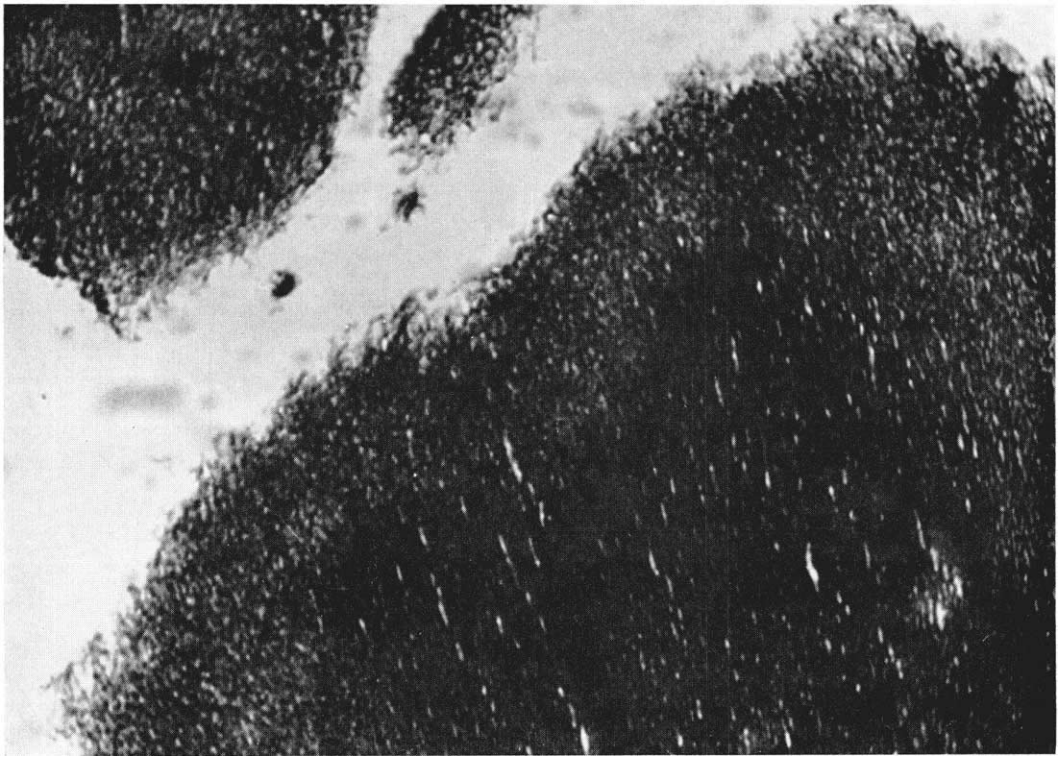

FIG. 9. "Granules" or microcolonies grown in TSB broth. Day 30. B. and B. Gram stain

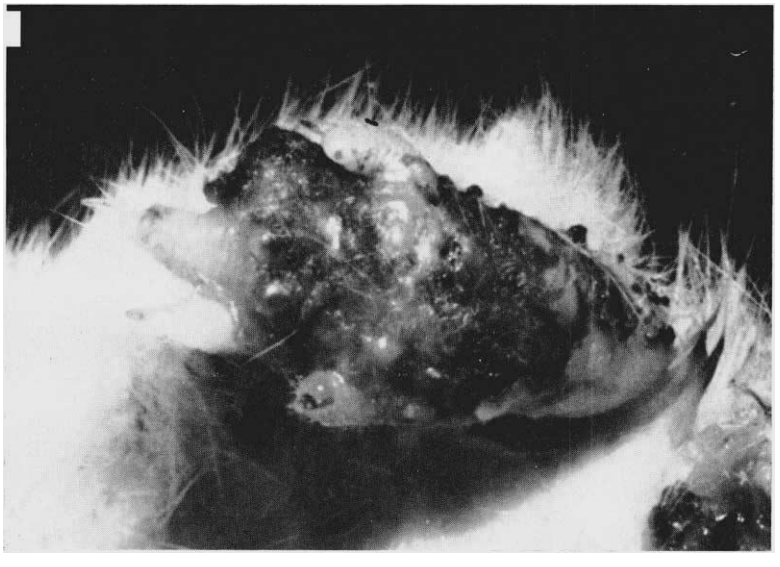

FIG. 10. "Madura foot" of mouse. Enlargement of leg and foot region with draining sinuses simulating human syndrome. Day 30.

TABLE II

Percentage survival of S. madurae *following exposure to NTG for varying times*

Time (min.)	5	10	20	30	60	90	120
Percentage survival	100	100	90	80	20	5	1.3

TABLE III

Collagenase production of twenty randomly selected clones following exposure to NTG

Collagenase activity (μM/mg*)	0	100–250	280–500	650–850	1000
Number of clones	2	3	5	9	1

* Average for wild type is 750.

production and lacking the ability to cause the complete disease syndrome in mice was treated with NTG. Thirty clones were randomly picked and tested for collagenase production. Colonies producing collagenase in amounts comparable to the wild type and identical to the wild type by all other criteria were tested for their ability to produce the disease. The extent and course of the disease were similar to those manifested by the wild type. No discernible differences between one virulent thoroughly tested revertant and the wild type were apparent (Table I).

DISCUSSION

An association between a specific toxin or enzyme and virulence has been proposed for several pathogenic bacteria, for example, toxigenicity in *Corynebacterium diphtheriae*. In the present work by using mutant strains, an association between collagenase-production and the ability to cause a disease has been demonstrated for *S. madurae* in experimental infections.

The inoculation of a virulent strain of *S. madurae* in mice elicits a mycetoma-like disease with granule formation and a histologic picture similar to that for the disease (madura mycosis) in humans. Furthermore, large areas of skin-loss occur which leads to death in most animals. A similar loss of skin and eventually death are observed when very low concentrations of collagenase are injected into normal animals (7).

Mutants of *S. madurae* unable to excrete a detectable quantity of collagenase initiated a disease but there was no associated skin loss and all inoculated mice survived. Revertants from a "collagenase-negative" strain produced disease and also caused the loss of skin. Finally, mutants producing greater quantities of collagenase than the original virulent strain were more virulent than the wild type organism. These observations strongly suggest a significant role for collagenase in the disease caused by *S. madurae* in experimental animals and a possible correlation with its pathogenicity in human disease.

Schoellman (3) has recently isolated *Pseudomonas aeruginosa* from a human ocular infection which was characterized by extensive damage to the collagen-structure of the cornea. The strain of *Pseudomonas aeruginosa* was shown to produce a collagenase.

SUMMARY

In experimental infection with normal collagenase-producing *Streptomyces madurae*, mycetoma-like grains were produced in mice with subsequent loss of vast areas of skin and high mortality. In mice injected with a collagenase-deficient mutant of *S. madurae*, granules were poorly formed and no skin loss or mortality occurred. Revertants to collagenase production had a virulence comparable to wild type organisms.

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